ENZYMIC CONVERSION OF AFLATOXIN B_1 TO A DERIVATIVE INHIBITING *IN VITRO* TRANSCRIPTION

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1. Introduction

Aflatoxin B_1, a verypotent hepatocarcinogen synthesized by *Aspergillus flavus*, strongly inhibits *in vivo* incorporation of labelled precursors into RNA of rat liver (see the review by Wogan [1]) by impairing the transcriptional mechanism of the cell [2–4]. However, the pure toxin fails to modify significantly the activity of an *in vitro* system composed of a DNA template and a RNA polymerase purified from bacteria [4, 5] or mammalian tissues [6]; by contrast, crude aflatoxin extracts prepared from *Aspergillus flavus* cultures are effective in inhibiting the RNA synthesizing system [4]. These results suggest that aflatoxin B_1 must be converted to a reactive molecule prior to interfering with cell metabolism. A similar sequence of events has been clearly demonstrated for other carcinogens (see the reviews by Farber [7] and Miller [8]).

This paper is related to the enzymic activation of aflatoxin B_1: using microsomal preparations of rat liver, we have obtained an aflatoxin-derived metabolite which is capable of inhibiting transcription *in vitro*. The mechanism of the inhibition has been studied. It has been shown that the active compound acts on RNA polymerase enzyme activity rather than on DNA template ability. Moreover, initiation appears to be the step of transcription affected by the active compound. The problem of the relationship between the carcinogenic potency of aflatoxin B_1 and the inhibitory effect of its derivative on *in vitro* transcription will be discussed.

2. Materials and methods

Mole Wistar rats (Commentry strain) weighing about 250 g received an intraperitoneal injection of 3-methylcholanthrene (20 mg per kg) dissolved in olive oil, for two consecutive days before killing. The livers were quickly removed and chilled at 0°; they were homogenized in 10 vol of ice-cold 0.25 M sucrose in a Perspex homogenizer. The homogenate was filtered through three layers of surgical gauze; the microsomal fraction was isolated according to Schneider [9]. Microsomes were resuspended in 0.05 M phosphate buffer pH 7.4 (microsomes from 1 g of liver per ml).

The enzymic conversion of aflatoxin B_1 was carried out at 37° in Erlenmeyer flasks. Each flask contained 100 μg aflatoxin B_1 dissolved in 0.2 ml acetone, 5 ml of 0.05 M phosphate buffer pH 7.4, 2.75 μmoles NADPH and 1 ml of microsomal suspension. The flasks were vigorously shaken for 30 min. The mixture was extracted three times with chloroform; the combined chloroform-soluble fractions were treated with Na_2SO_4 and evaporated to dryness. The residue was dissolved in either propylene glycol or Tris-buffer.

Thin-layer chromatography was performed on silica gel G-HR (Macherey Nagel) coated plates using chloroform:acetone (9:1) for development.

The RNA polymerase system contained in 0.25 ml: 10 μmoles Tris-buffer pH 9, 0.25 μmoles $MnCl_2$, 1.25 μmoles $MgCl_2$, 3 μmoles β-mercaptoethanol, 80 μg calf thymus DNA (unless otherwise stated), 0.025 μmoles each of three unlabelled nucleoside-5′-tryphosphates (ATP, GTP CTP), 0.025 μmoles of [2-^{14}C]UTP corresponding to 0.09 μCi, *E. coli* RNA polymerase.

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Table 1

Effect of aflatoxin B_1 derived metabolite (compound X) on the activity of an RNA synthesizing system using bacterial polymerase.

		Number of preparations and quantity of "compound X" added to the assay				
	Sequential addition of components to transcription assay	X_2 0.5 μg	X_{4a} 0.4 μg	X_7A_1 0.75 μg	X_7C_1 0.6 μg	Aflatoxin B_1^* 10 μg
Part a	DNA + NTP (2 min at 37°) + enzyme, 10 min at 37°	(100)	(100)	(100)	(100)	(100)
	DNA + compound X + NTP (2 min at 37°) + enzyme, 10 min at 37°	50	58	43	70	~ 100
Part b	Enzyme + DNA (2 min at 37°) + NTP, 10 min at 37°			(100)	(100)	
	Enzyme + NTP + compound X (2 min at 37°) + DNA, 10 min at 37°			44	25	
	Enzyme + DNA (2 min at 37°) + compound X + NTP, 10 min at 37°			66	49	
Part c	DNA + NTP (2 min at 37°) + enzyme, 2 min at 37° + compound X, 8 min at 37°			~ 100	80	

The composition of the assay was described in Materials and methods. Aflatoxin B_1 and compound X_2 were dissolved in propylen glycol (0.05 ml); compounds X_{4a}, X_7A_1 and X_7C_1 were in solution in 0.01 M Tris-HCl buffer pH 7.8. The amounts of RNA polymerase enzyme used corresponded to 5.8 units (25 μg protein). * Compound X was replaced by 10 μg aflatoxin B_1.

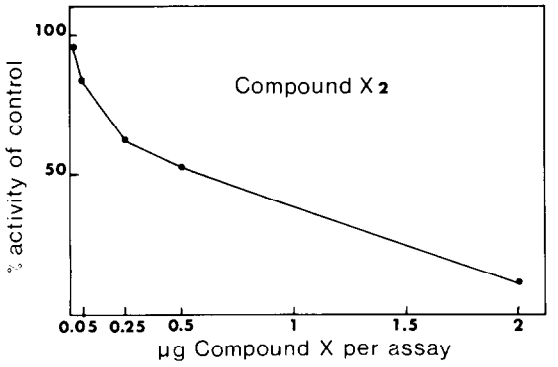

Fig. 1. Inhibition of RNA polymerase activity in the presence of progressively higher amounts of compound X. The RNA polymerase assay is described in Materials and methods. The amount of RNA polymerase used corresponded to 5.8 units (25 μg protein). Compound X_2 was dissolved in 0.05 ml of propylene glycol.

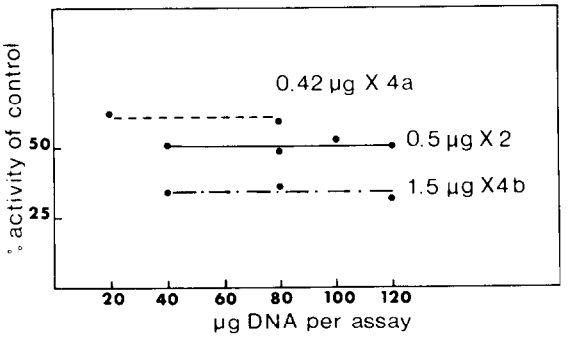

Fig. 2. Effect of progressively higher amounts of DNA on the inhibition of *in vitro* transcription by compound X. Compound X_2 was dissolved in 0.05 ml of propylene glycol; compounds X_{4a} and X_{4b} were in 0.01 M Tris-HCl buffer pH 7.8. The amount of RNA polymerase corresponds to 5.8 units (25 μg protein).

Aflatoxin B_1 or the aflatoxin-derived compound dissolved in propylene glycol or Tris-buffer pH 7.5 was added in the amounts indicated in the figures. Controls contained the same volume of propylene glycol or Tris-buffer. The reaction was run in duplicate for 10 min at 37°. Determination of incorporated radioactivity was performed as described previously [10].

Purified RNA polymerase from *E. coli* was purchased from Miles Laboratories (Elkart, Indiana, USA) and aflatoxin B_1 from Makor (Jerusalem, Israel).

3. Results

Chloroform-soluble compounds, obtained by incubating aflatoxin B_1 in the presence of microsomes, were subjected to thin-layer chromatography on silica gel coated plates. A new blue fluorescent spot appeared with an R_f lower than that of aflatoxin B_1. This new component was called "compound X".

The new fluorescent fraction was isolated by chromatography of chloroform-soluble compounds through a silica column followed by elution with chloroform: ethanol (98.75 : 1.25). Compound X was estimated by fluorodensitometric measurement using the specific coefficient of aflatoxin B_1. The absolute amounts of compound X reported in this paper should be corrected when its identification will have been done, allowing the determination of its own emission coefficient. This problem is under investigation.

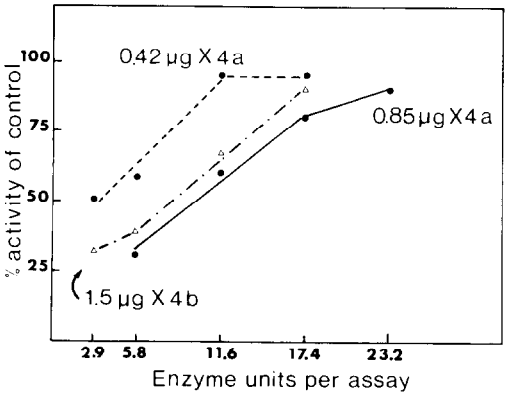

Fig. 3. Effect of progressively higher amounts of *E. coli* RNA polymerase on the inhibition of *in vitro* transcription by compound X. Compounds X were in solution in Tris-HCl buffer 0.01 M pH 7.8; each assay contained 80 µg calf thymus DNA.

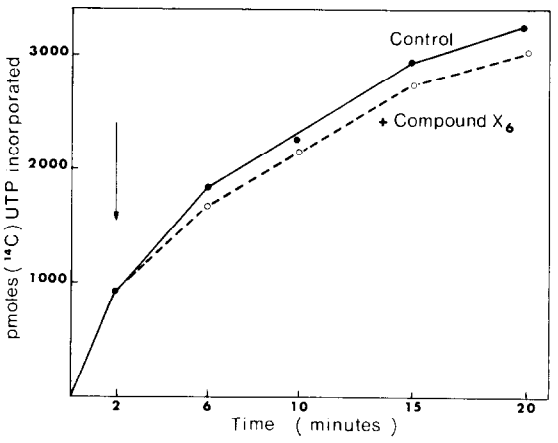

Fig. 4. Time course incorporation of [^{14}C]UTP in an RNA polymerase system in the presence of compound X added after initiation of the reaction. Compound X_6 (2.6 µg) was dissolved in acetone (0.02 ml). At the time indicated by the arrow, control assays received 0.02 ml of acetone and the other series received 2.6 µg of compound X_6 in solution in 0.02 ml acetone; the assays were run for a further incubation and stopped as indicated in the figure. Addition of 0.02 ml acetone in the controls induced an 18% inhibition of the activity of assays performed without acetone.

Compound X markedly inhibits the *in vitro* RNA-synthesizing system, whereas aflatoxin B_1 is completely inactive (table 1, parts a and b). Although we observed some differences in the activity of the successive preparations of the aflatoxin-derived fraction (see Discussion) in most cases a 50% inhibition as compared to the control value was obtained for 0.5 to 0.8 µg of compound X, a significant inhibition was observed for a dose as low as 0.05 µg for the most active preparation (fig. 1). However, whatever the batch of compound X the extent of inhibition always depends on the amount of compound X added.

Further experiments were performed in order to provide information regarding the site of action of the aflatoxin-derived metabolite. Results reported in fig. 2 show that the extent of inhibition of transcription is unaffected by increasing amounts of DNA template added in the assay regardless of whether DNA is a limiting or a non-limiting factor for the reaction. By contrast, addition of higher levels of RNA polymerase (2.9 to 23.2 units) relieves progressively the inhibitory effects on RNA synthesis (fig. 3). These facts suggest that compound X impairs *in vitro* transcription by alteration of the RNA polymerase enzyme itself.

Regarding the step of the transcriptional process affected, the results indicate that inhibition concerns primarily initiation since addition of compound X 2 min after the reaction had started does not block the further elongation of chains initiated before action of the drug (table 1, part c). The time course of the incorporation of labelled UTP into polynucleotides (fig. 4) is similar to that observed for rifampicin and rifampicin AF/0.13, two drugs which are known to inhibit specifically the initiation of chains, respectively, in bacterial [11] and mammalian systems [12]. This conclusion is strengthened by the fact that compound X fails to inhibit transcription carried out on isolated liver nuclei. Under these conditions it is known that no initiation of new chains takes place *in vitro*: RNA polymerase activity corresponds only to elongation of chains preinitiated.

4. Discussion

The enzymes involved in the metabolic detoxification by liver are tightly associated with microsomal membranes and are induced in response to administration of hormones, drugs and carcinogens (see Brodie et al. [13] and Talalay [14]). Enhanced enzymic capacity of microsomes obtained from rats treated with methylcholanthrene [15] was used to achieve *in vitro* conversion of aflatoxin B_1. By this technique, a fraction containing a new fluorescent component (compound X) derived from the toxin was obtained. Unlike aflatoxin B_1, it inhibits *in vitro* activity of *E. coli* polymerase. Further experiments will determine the action of this fraction on mammalian purified RNA polymerase.

Depending on the preparations, the inhibitory capacity of compound X shows some differences. However, despite the quantitative variations, successive batches of compoun X have a reproducible action on the molecular mechanism of the transcription process (table 1; figs. 2 and 3). The foregoing results suggest that the compound X have a reproducible action on spond to more than one molecular species, the relative amount of the true active metabolite varying from one batch to the other. Thin-layer chromatography of compound X only shows one blue fluorescent spot. However, it should be pointed out that the chromatographic behavior of a compound mainly depends on the number of polar groups; thus, this procedure may fail to separate isomeric compounds having the same polarity whatever their fluorescence property.

Attemps are in progress to obtain a better isolation of the active metabolite in collaboration with Jemmali and Lauriere. This step will permit identification of the active molecule. In this search, attention must be paid to some well-known derivatives that have to be seriously considered such as aflatoxin M_1 [16, 17] and aflatoxin P[18] which correspond, respectively, to the hydroxylated and demethylated forms of aflatoxin B_1. Recent results by Pong and Wogan [19] have demonstrated that both aflatoxin B_1 and M_1 induce similar acute effects on RNA metabolism *in vivo* in rat liver. However, the effect of M_1 on transcriptional activity *in vitro* has not yet been reported.

Compound X appears to inhibit *in vitro* transcription by acting on RNA polymerase rather than on DNA template (figs. 2 and 3). This does not exclude the possibility of an *in vitro* effect of the active metabolite on DNA but indicates that its interaction with the protein-enzyme must also be considered to explain alterations in the catalytic properties of the RNA synthesizing system.

It has been clearly demonstrated that many chemical carcinogens are not carcinogenic as such; they must be converted to reactive intermediates or ultimate forms which actually possess the carcinogenic potency [7, 8]. In fact, it has been thought that only the ultimate forms bind to DNA in physiological conditions, that predicts a potential mutagenic property.

It has been shown that aflatoxin B_1 is capable of interacting with calf-thymus DNA *in vitro* [20] but there is no rigorous proof demonstrating that such a complex occurs *in vivo*. On the other hand, some mutagenic action of purified aflatoxin B_1 on transforming DNA has been reported by Mahler and Summers [21].

Aflatoxin B_1 is completely inactive on *in vitro* transcription [4–6] and translation (manuscript in preparation) in mammalian systems, whereas compound X does inhibit their activity. It would be of interest to determine whether compound X resulting from enzymic conversion of aflatoxin B_1 might represent the true reactive carcinogen. If so, a study of the interaction of compound X with macromolecules at the cellular level in the target cell may contribute to a better understanding of the mechanism of carcinogenesis induced by aflatoxin B_1.

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